REMARKS

I. Introduction

In response to the Office Action dated January 25, 2005, claims 1-4, 6, 7 and 9 have been canceled, and claims 5, 8 and 10-13 have been amended. Claims 5, 8 and 10-13 remain in the application. Reconsideration of the application, as amended, is requested.

II. Claim Amendments

Applicants' attorney has made amendments to the claims as indicated above. These amendments were made solely for the purpose of clarifying the language of the claims, and do not introduce new matter. Entry of these amendments is respectfully requested.

Claim 5 has been amended to recite "isolated", and to clarify the recitation of the nucleotide sequence.

Claim 8 has been amended to place it in independent form, and to clarify the recitation of the nucleotide sequence.

Claim 10 has been amended to update the reference to preceding claim 8, rather than cancelled claim 6.

Claims 11-13 have been amended to clarify the reference to claim 10, from which these claims depend.

III. Sequence Rules

At page 2 of the Office Action, it was noted that the application fails to comply with the Sequence Rules because sequences recited in the Figures are not identified in the specification with appropriate indicators. In response, Applicants have amended the Brief Description of the Figures at pages 8-9 of the substitute specification (clean form; page 9 of the marked-up version), to introduce appropriate SEQ ID NO: indicating the corresponding sequences shown in Figures 1 and 2.

IV. Substitute Specification

At page 2 of the Office Action, it was noted that the specification is an English translation of the Korean application, but is not in proper idiomatic English. Submission of a substitute specification, placing the specification in proper idiomatic English was requested. Such a substitute specification is submitted herewith, in both marked-up and clean forms. This substitute specification also includes amendment to pages 8-9, to introduce SEQ ID NO: 15-20, corresponding to the sequences appearing in Figures 1 and 2. Applicants hereby state that the substitute specification filed herewith contains no new matter. This statement is also provided as a separate document submitted herewith, in compliance with 37 CFR §1.125(b).

V. Non-Art Rejections

At page 5 of the Office Action, claims 1-5 were rejected under 35 U.S.C. §101 because the claimed invention is directed to non-statutory subject matter. The cancellation of claims 1-4 renders the rejection of these claims moot. Claim 5 has been amended to recite "isolated", as suggested by the Examiner, to overcome this rejection.

VI. Prior Art Rejections

At page 3 of the Office Action, claims 1-4, 6, 7, 9-13 were rejected under 35 U.S.C. §102(b) as being anticipated by Kim et al. The cancellation of claims 1-4, 6, 7 and 9 renders the rejection of these claims moot. Claims 9-13 have been amended to depend from claim 8, which is free of the art.

VII. Allowable Subject Matter

At page 5 of the Office Action, claims 5 and 8 were indicated as free of the art because the *P. rhodozyma* ribosomal DNA sequence recited in the claims (SEQ ID NO: 4) is not taught by the prior art. Claim 8 was objected to as being dependent upon a rejected base claim, but allowable if rewritten in independent form, including all of the limitations of the base claim and any intervening claim. Applicants have amended the claims accordingly, to place them in condition for allowance.

VIII. Conclusion

In view of the above, it is submitted that this application is now in good order for allowance and such allowance is respectfully solicited. Should the Examiner believe minor matters still remain that can be resolved in a telephone interview, the Examiner is urged to call Applicants' undersigned attorney.

Respectfully submitted,

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VECTOR FOR THE TRANSFORMATION OF *Phaffia rhodozyma* AND PROCESS OF TRASFORMATION THEREBY

FIELD OF THE INVENTION

The present invention is directed~~relates~~ to a novel vector~~s~~ for ~~the~~ transforming~~ation~~ ~~*of Phaffia rhodozyma*~~ yeast and to a process for ~~of~~ transforming yeast~~ation~~ thereby. Particularly, the present ~~this~~ invention is directed~~relates~~ to a~~n~~ ~~L41~~ gene encoding L41, a ribosomal protein derived from *Phaffia rhodozyma* which is useful for producing natural pigment astaxanthin; a~~n~~ ~~L41~~ gene encoding a mutated L41 protein~~mutated to~~having a cycloheximide-resistant activity~~form~~; a ribosomal DNA derived from *Phaffia rhodozyma*~~ ribosomal DNA~~; a vector for transforming ~~the stable transformation of~~ *Phaffia rhodozyma*~~,~~ stably, comprising said gene encoding a mutated L41 protein ~~mutated L41 gene~~ and said ribosomal DNA; and a process ~~of~~ for transforming~~ation~~ *Phaffia rhodozyma* thereby.

BACKGROUND

Phaffia rhodozyma is a reddish yeast species producing astaxanthin, ~~the~~ a useful natural pigment. Astaxanthin is a member of the carotenoids, which are represented by β-carotene, ~~the~~ a precursor of vitamin A. ~~Naturally,~~ A~~a~~staxanthin as a main pigment of curstacea, trout and salmon is widely distributed in nature~~,~~. ~~especially to Crustacea, trout and salmon as their main pigment,~~ However, ~~although~~ they cannot synthesize astaxanthin and should be supplied with it from ~~the~~ a diet. Thus, it has been considered

necessary to add the pigment in the cultivation of cCrustacea, trout and salmon, ~~so that~~because the added pigments to the cCrustacea and fishes may attract the consumers and give better flavors to them. This carotenoid pigment plays key roles in the physiological metabolism of human as well as animals, with known effects such ~~as the precursor of vitamin A, the~~ an enhancement of immunological function, ~~the~~ an antioxidant activity, ~~the~~ a prevention of cancer and senescence, etc.

Because of increasing interests in *Phaffia rhodozyma* and pigments produced thereby, there have been a number of reports ~~concerned about~~concerning a ~~the~~ culture of *Phaffia rhodozyma*. However, these ~~researches~~ reports have been focused on how the inexpensive materials can be used for its culture, and have resulted in the development of method for culturing *Phaffica rhodozyma*~~culturing methods~~, in which various local products may be employed, such as alfalfa juice (Okagbue *et al.*, *Appl. Microbiol. Biotechnol.*, 20, 33, 1984), molasses (Haard *et al.*, *Biotechnol. Lett.*, 10, 609, 1988), the byproducts of grape juice processing (Lango *et al.*, *Biotech. Forum Europe*, 9, 565, 1992), peat hydrolyzate (Martin *et al.*, 58, 223, 1993), the byproducts of corn wet-milling (Hayman et al., *J. Ind. Microbiol.*, 14, 389, 1995), and the mixture of sugar cane extract, urea and phosphoric acid (Fontana, *et al.*, *Appl. Biochem. Biotechnol.*, 57/58, 413, 1996).

Although little is known about the genetics of *Phaffia rhodozyma*, the physiological features of

Phaffia rhodozyma have been disclosed and the *Phaffia rhodozyma* mutant producing the pigment with high level has recently been selected ~~to produce higher level of the pigment~~ (Johnson *et al.*, *Crit. Rev. Biotechnol.*, 11, 297, 1991; An *et al.*, *Appl. Environ. Microbiol.*, 55, 116, 1989; Chumpolkulwong *et al.*, *J. Ferment. Bioeng.*, 75, 375, 1997; Lewis *et al.*, *Appl. Environ. Microbiol.*, 56, 2944, 1990). In addition, a genetic analysis enlightened the ploidy and sexual cycle of *Phaffia rhodozyma*. In a flow cytometry study, Calo-Mata and Johnson found that no strain was haploid and that most were polyploid (Calo-Mata *et al.*, *Yeast Gen. Mol. Biol. Meet.*, 126, 1996). A pedogamic sexual process of conjugation has been also desclosed~~ribed~~ (Golubev *et al.*, *Yeast*, 11, 101, 1995).

Although *Phaffia rhodozyma* is potentially useful for the production of astaxanthin and the like, the pigment level in the wild type of *Phaffia rhodozyma* is very low. Therefore, there have been ~~increasing~~ attempts to develop a novel mutant strain of *Phaffia rhodozyma*, which can produce ~~the higher level of~~ the pigment more than usual one. However, these attempts have been hampered by the reduced growth rate and genetic instability of said mutant, which may occur when the pigment content in ~~a~~ the mutant exceeds over the optimal range.

Another obstacle to the progress of the mutant is the method ~~of~~ for mutagenesis. Chemical mutagenesis procedures ~~has~~ have been performed conventionally, but it is associated with the simultaneous mutation of

undesired genes leading to pleiotropic effects such as the reduction of growth rate, the ~~prolongation~~ prolonged~~of~~ induction time in the fermentation, etc. Furthermore, because the genome of the mutant strain is not stable, ~~since~~ its subculture often decreases the yield of the pigment.

To solve these problems in the conventional breeding procedures and to enlarge the applicability of *Phaffia rhodozyma*, molecular breeding approaches have been initiated recently, using genetic transformation. However, since most of *Phaffia rhodozyma* strains are polyploid and thus cannot be made to be an auxotrophic variant by the method conventionally applied to yeast, it is preferable to employ an ~~preferable is the~~ approach using ~~employing~~ antibiotics-resistant genes as selectable markers. More recently, there was reported a transformation system in which *Phaffia rhodozyma* actin promoter and G418-resistant gene were used for the transformation of *Phaffia rhodozyma*~~,~~. However, ~~although~~ the system ~~it~~ showed poor transformation efficiency (Wery et al., Gene, 184, 89, 1997).

On the other hand, cycloheximide, an eukaryote-specific antibiotics, is applicable to the selection of yeast transformants. The target molecule of cycloheximide is ribosome and its target site ~~action~~ is aminoacyl-tRNA binding site (A site) of ribosome, wherein it blocks peptidyl transferase activity of ribosome. As a result, it inhibits protein synthesis and cell growth in eukaryotes, without an effect on the

organelles such as chloroplasts and mitochondria. Furthermore, it has been found that cycloheximide interacts with ribosomal protein L41, and that a mutation in L41 gene confers cycloheximide-resistance on the yeast transformants. Thus, cycloheximide and related mutant form of L41 gene are widely applicable to the process ~~of~~ for transformation ~~for~~ of yeasts.

Recent studies support the applicability of L41 gene to selectable marker in yeasts. Takagi et al. found that amino acid substitution through the mutagenesis of *Saccharomyces cerevisiae* L41 gene conferred cycloheximide-resistance, suggesting the usefulness of L41 gene as a selectable marker (Takagi *et al.*, *J. Bacteriol.*, 174, 254-262, 1992). Mutoh et al. proposed a biotechnological tool using *Candida maltosa* L41 gene as a selectable marker (Mutoh et al., *J. Bacteriol.*, 5383, 177, 1995). As it is well known that a substitution of 56^{th} amino acid residue in the L41 protein conffer cycloheximide-resistant on Candida utilis ~~cycloheximide-resistance is conferred on *Candida utilis* as well as *Phaffia rhodozyma* by the substitution of 56th amino acid residue in the L41 protein~~ (Keiji Kondo *et al.*, *J. Bacteriol.*, 7171, 177, 1995), transformation system using the substitution ~~thereby~~ has been developed. Similar approaches have been introduced ~~attempted~~ in *Kluyveromyces lactis* and *Schwanniomyces occidentalis* (Dehoux *et al.*, *Eur. J. Biochem.*, 213, 841-843, 1993; Pozo *et al.*, *Eur. J. Biochem.*, 213, 849-857, 1993). On algae *Tetrahymena*, the resistance is conferred by substitution of 40th amino acid residue, methionine to glutamine (Roberts *et*

al., Exp. Cell. Res., 312, 81, 1973).

To overcome the foregoing and other disadvantages, we, the inventors of the present invention, have noted that cycloheximide and related mutation in L41 gene may be used to develop an efficient transformation system, whereby~~in which~~ a foreign gene is stably integrated into the genome of *Phaffia rhodozyma*, and ~~in which~~ the transformants are undoubtedly selected. To develop such system, we have constructed transforming vectors comprising the antibiotics-resistant gene and the targeting gene, which is used for the stable integration of foreign gene. We transformed *Phaffia rhodozyma* with ~~such~~ asid vectors, according to a modified method for electrotransforming *Cryptococcus neoformans*, a member of Basidiomycetes, ~~of which~~whereto *Phaffia rhodozyma* belongs~~is also another member~~ (Kim *et al., Appl. Environ. Microbiol.*, 64, 1947, 1998).

The present invention is performed by cloning and sequencing *Phaffia rhodozyma* L41 gene; modifying the L41 gene by the mutagenesis of the region responsible to cycloheximide-resistance; constructing the vectors for transforming by inserting ribosomal DNA into the ~~mutated~~ modified L41 gene; transforming *Phaffia rhodozyma* with the vector by electroporation method; and verifying the stable integration of the vector into the genome of the transformants.

SUMMARY OF THE INVENTION

It is an object of ~~this~~ the present invention to provide a vector for transforming *Phaffia rhodozyma*

efficiently.

It is a further object of ~~this~~ the present invention to provide a~~an~~ recombinant~~antibiotics-resistant~~ vector for transforming *Phaffia rhodozyma*, which comprises the L41 protein of *Phaffia rhodozyma*.

It is an additional object of this invention to provide a L41 gene encoding the L41 protein of *Phaffia rhodozyma*~~.~~, which has an antibiotics-resistant activity.

It is another object of this invention to provide a ~~mutated L41~~ gene encoding a mutated L41 protein ~~that~~which can be used as a cycloheximide-resistant gene.

It is still another object of ~~this~~ the present invention to provide a ribosomal DNA of *Phaffia rhodozyma*, which can be used to enhance the integration efficiency of foreign DNA into *Phaffia rhodozyma* genomes.

It is ~~also~~ another object of ~~this~~ the present invention to provide a process ~~of~~ for transforming *Phaffia rhodozyma* by electroporation.

Further objects and advantages of the present invention will appear hereinafter.

In accordance with the present invention, the foregoing objects and advantages are readily obtained.

The present invention provides an L41 gene encoding a ribosomal protein ~~originated~~ derived from *Phaffia rhodozyma*.

In addition, this invention provides a ~~mutated L41~~ gene encoding mutated L41 protein~~ gene in which~~wherein

the amino acid at the position 56 is replaced by glutamine. Since the amino acid residue is responsible for the cycloheximide-resistance, this mutated gene in a vector is useful for a selectable marker.

The present ~~This~~ invention also provides a ribosomal DNA derived from *Phaffia rhodozyma*.

In addition, the present ~~this~~ invention provides a recombinant vector comprising a gene encoding a protein having a cycloheximide-resistant ~~cycloheximide-resistant gene~~ activity and a ribosomal DNA derived from *Phaffia rhodozyma*.

In such aspect of ~~this~~ the present invention, also provided is a recombinant vector, pTPLR1 comprising ~~the~~ a ~~mutated~~ gene encoding the muated L41 ~~gene~~protein of *Phaffia rhodozyma* and a portion of the *Phaffia rhodozyma* ribosomal DNA.

~~This~~ The present invention also provides a process of transforming *Phaffia rhodozyma* with the vector by electroporation.

In a preferred embodiment~~such aspect~~ of the present ~~his~~ invention, the vector is ~~preferably~~ cleaved into a linear form~~,~~. In another preferred embodiment of the present invention, the linearized vector is tintroduced into *Phaffia rhodozyma* using electrophoresis. In the more preferred embodiment of the present invention~~and the preferable condition for~~ the electrophoresis is conducted with conditions as follows: ~~electroporation is such that~~ electric pulse is 0.8-1.2 kV~~,~~; an internal resistance is 400-800 Ω;~~,~~ and a capacitance is 25-50 μF.

Further features of the present invention will

appear hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is nucleotide sequence of genomic DNA containing upstream promoter region and coding region of a gene encoding L41 ribosomal protein of *Phaffia rhodozyma* (SEQ ID NO: 15) and deduced amino acid sequences ~~of~~by the gene (SEQ ID NO: 16) ~~L41 gene encoding *Phaffia rhodozyma* ribosomal protein~~, wherein,

Open boxes: TATA and CAAT sequences;

Underlined: the position of primers;

Bold letters: consensus sequence in splicing region of intron;

Open circle: amino acid residue at position 56

Figure 2 represents the construction of the pTPLR1 vector, ~~and~~ its restriction map, a nucleotide sequcence for mutagenesis of L41 gene(SEQ ID NO: 17) and its amino acid sequence (SEQ ID NO: 18) and a mutated nucleotide sequence (SEQ ID NO: 19) and its amino acid sequence, wherein,

Numbers in parentheses: the sizes of inserts;

Blank boxes: DNA fragment containing L41 gene;

Grey boxes: rDNA fragments;

Black boxes: exons of L41 gene;

Thin lines: pBluescript SK(+) sequence;

Horizontal arrow: transcriptional direction of L41 gene;

X: *Xba*I site; S: *Sal*I site; C: *Cla*I site;

H: *Hind*III site; E: *Eco*RI site; Xh: *Xho*I

site;

Sm: *Sma*I site; Bg: *Bgl*I site; Ba: *Bal*I site;

Kp: *Kpn*I site;

Figure 3 represents the restriction map of pTPLR1, the vector of ~~this~~ the present invention,

Figure 4 represents the relationship between the condition of electroporation and the transformation efficiency or cell viability;

Figure 5 represents Southern blot analysis of pTPLR1 transformants, wherein,

C: nontransformant control;

1 to 5: pTPLR1 transformants;

Figure 6 is a ~~represents~~ schematic~~ally~~ diagram showing the mode of pTPLR1 integrated into the chromosome.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention is based upon the notion that cycloheximide and related mutation in a gene encoding L41 ~~gene~~protein may be used to develop a transformation system, ~~in which~~whereby a foreign gene is stably integrated into the genome of *Phaffia rhodozyma*, and ~~in which~~ the transformants are undoubtedly selected.

Hereinafter, the present invention is described in

detail.

In one aspect, the present invention provides a ~~L41~~ gene encoding a L41 *Phaffia* ribosomal protein.

In a preferred embodiment, ~~we have obtained~~ the genomic and cDNA sequences containing ~~the L41~~ a gene encoding a L41 *Phaffia rhodozyma* ribosomal protein~~, and these sequences~~ are prepared from a *Phaffia rhodozyma* strain (ATTC 24230).

The ~~L41~~ gene encoding L41 protein identified in the present ~~this~~ invention shows high homology with other known L41 gene ~~of~~derived from yeasts, but contains 6 introns which have specific sequences in 5' and 3' regions of each intron. The genomic sequence represented ~~described~~ by SEQ ID NO: 1 contains 7 exons and 6 introns and the cDNA ~~the~~ encoding the L41 protein ~~gene of~~ has a nucleotide sequence of 1,223 bp fragment ~~, which in turn contains the cDNA sequence~~ represented ~~described~~ by SEQ ID NO: 2. ~~Of the~~ The deduced amino acid sequence is ~~described by~~ SEQ ID NO: 3~~,~~. The proline at position 56 is responsible for ~~the~~ sensitivity to cycloheximide (see FIG 1).

In another preferred embodiment, the cloned gene encoding L41 protein~~gene~~ is modified by site-directed mutagenesis, so that the mutated L41 protein~~gene~~ has ~~is made to be a~~ cycloheximide-resistant activity~~gene, or gene which can confer resistance to cycloheximide on an acceptor organism~~. Particularly, ~~the~~ a mutagenesis is performed to substitute ~~replace~~ the proline residue with ~~by~~ glutamine, at the position 56 (see FIG 2).

The mutagenesis in the present ~~this~~ invention includes all the possible modification of triplet codon

in the amino acid position 56. For example, the codons for proline 56 may be replaced by all possible triplet codons for glutamine.

~~This~~ The present invention also provides a ribosomal DNA (hereinafter "rDNA") derived from *Paffia* yeast.

In this invention, rDNA means not only a DNA sequence which is transcribed to bear all types of eukaryotic ribosomal RNA, but also a non-transcription spacer (hereinafter, "NTS"), or a DNA sequence between the transcribed rDNA. rDNA can be preferably used to enhance the integration efficiency of foreign DNA into host genomes because rDNA sequence is highly repeated as tandem units in the eukaryotic genomes.

In a preferred embodiment, ~~we identified~~ the rDNA ~~which~~ is ~~described~~ represented by SEQ ID NO: 4. ~~This~~ The rDNA sequence contains NTS.

The present~~This~~ invention provides a ~~transforming~~ recombinant vector for transforming Phaffia rhodozyma, comprising a cycloheximide-resistant gene and a rDNA.

According to one preferred embodiment, the cycloheximide-resistant gene is a gene coding a mutated L41 protein derived *Phaffia rhodozyma.*

According to one preferred embodiment, the rDNA may be used to enhance the integration efficiency of foreign DNA into the host genome.

According to more preferred embodiment, the rDNA has a sequence of SEQ ID NO: 4.

According to another preferred embodiment, the

gene encoding L41 protein of *Phaffia rhodozyma* ~~L41 gene~~is~~—~~ modified so as to have a ~~to~~ cycloheximide-resistant acitivity~~gene~~ and be used~~—is employed~~ as a selectable marker in the ~~transforming~~recombinant vector (see fIG 2). This ~~transforming~~recombinant vector is useful for a~~the~~ stable transformation~~introduction~~ of a foreign gene into a host genome.

More particularly, the ~~his~~ present invention provides pTPLR1, a vector for transforming yeasts~~,~~. In the most preferred~~ably~~ ~~for transforming Phaffia rhodozyma,~~ embodiment the recombinant vector ~~which~~ comprises an NTS portion of *Phaffia rhodozyma* rDNA and a gene encoding a mutated L41 protein of *Phaffia rhodozyma* ~~L41 gene~~ wherein the codon for proline at amino acid position 56 is ~~replaced~~ substituted with~~by~~ the codon for glutamine (see FIG 3).

The recombinant~~transforming~~ vectors of the present ~~this~~ invention may be readily modified and improved within the spirits and scope of the present ~~this~~ invention. For example, the recombinant~~transforming~~ vector of the present ~~this~~ invention may include diverse L41 genes modified ~~through~~ using various mutagenesis procedures and diverse rDNA sequences derived ~~originated~~ from various organisms.

In another aspect of ~~this~~ the present invention, also provided is a process ~~of~~ for transforming yeasts with foreign DNA. The process is based upon the established method for transforming *Cryptococcus neoformans*, but optimized to yeasts, using an antibiotics-resistance gene derived from yeasts instead

of the bacterium-derived counterpart.

In a preferred embodiment, the ~~transforming~~recombinant vector is cleaved into a linear form before transformation. The restriction enzymes used and the reaction may be selected carefully so that the foreign DNA is efficiently introduced into a host genome and only desired sequences of the vector are inserted to the host genome.

In the ~~transforming~~ process for transforming of ~~this~~ the present invention, an electroporation procedure is employed. According to another embodiment, ~~the preferable condition for~~ electroporation is conducted with conditions as follows: ~~is such that~~ an electric pulse of~~is~~ 0.8~1.2 kV, an internal resistance ~~is~~ of 400~800 Ω, and a capacitance ~~is~~of 25~50 μF. After electroporation, the yeast cells are cultivated~~ured~~ at 23°C for 14~16 hours, then spread on solid medium containing cycloheximide, and further cultivated~~ured~~ at 23°C for 4~5 days. Assessing the effects of various conditions for the electroporation on the cell viability and the transforming efficiency (see FIG 4) reveals that abundant transformants are produced under such condition as electric pulse of 0.8 kV, an internal resistance of 600 Ω, and a capacitance of 50 μF.

In ~~still~~ another embodiment, Southern blot analysis is used to verify the stable integration of foreign DNA (see FIG 5 and 6). The result confirms that the introduced genes are stably maintained in host genome, even after multiple subcultures on the medium without cycloheximide.

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: The isolation of gene encoding a L41 protein of~~-~~ *Phaffia rhodozyma*~~-L41-gene~~

To isolate genomic DNA sequence encoding *Phaffia rhodozyma* ribosomal protein L41, we synthesized two PCR (~~;~~ polymerase chain reaction) primers, the sequences thereof ~~which~~ were deduced from the nucleotide sequence of other yeast L-41 genes and represented~~described~~ by SEQ ID NO: 5 (CYH1) and SEQ ID NO: 6 (CYH3). PCR was performed ~~in which~~ using the synthetic oligonucleotides, CYH1 and CYH3 ~~were used~~ as PCR primers and ~~in which~~ genomic DNA isolated from *Phaffia rhodozyma* (ATCC 24230) ~~was employed~~ as template. A DNA fragment of 700 bp containing a gene encoding L41 protein was ~~The PCR~~ produced ~~700 bp DNA fragments containing L41 gene~~ as a result, and~~which were~~ then was brought to ~~the~~ a labeling reaction using digoxigenin (DIG)-labeling kit (Boehringer Mannheim, Germany) so as to be used as a probe for Southern blot analysis. To clone full-length genomic DNA encoding L41 ~~gene~~protein, Southern blot hybridization was performed as des~~cribed~~closed ~~in the work of~~ by Sambrook et al.

(Sambrook et al., Molecular Cloning, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989) in a solution containing 5X SSC, 0.1% (w/v) sarcosyl, 0.02% (w/v) SDS, 5% blocking agent, and 50% (v/v) formamide, at 42°C. A strong hybridization signal was observed from an 8-kb *Xba*I fragment, and the *Xba*I fragments of 7 to 9-kb were isolated and ligated into pBluescript SK(+) (Stratagene, USA) to make a minilibrary. A clone (pTPL2), hybridizing with the PCR product was identified in a further Southern blot analysis ~~in which~~wherein the DNA fragment~~s~~ of the minilibrary were blotted onto the membrane.

To identify the gene encoding L41 ~~gene~~protein without intron, *Phaffia rhodozyma* L41 cDNA was isolated by the method of rapid amplification of cDNA ends (~~,~~ RACE) with 3'-RACE (GIBCO BRL, USA) and 5'-RACE (Clontech, USA) kits. Total RNA was prepared by the method of Elion and Warner (Elion *et al.*, *Cell*, 39, 663-673, 1984). Then mRNA was selected from the total RNA, using mRNA isolation kit (Novagen), and ~~brought to~~ a 3' RACE reaction was performed with ~~in which~~ a synthetic oligonucleotide ~~described~~ represented by SEQ ID NO: 7 ~~was used~~ as a 3' RACE primer, and 5' RACE reaction was ferformed with a synthetic oligonucleotide represented by SEQ ID NO: 8 as a 5' RACE primer.

The sequencing of the 3' and 5' RACE products suggested that a putative open reading frame of 1,223 bp be interrupted by six introns. The cloned gene encoding L41 ~~gene~~ protein was found to show high homology with those of other yeasts. However, the number of introns and their organization in the gene

encoding the L41 protein of *Phaffia rhodozyma* ~~L41 gene~~ were quite different from those of the other yeast ~~L41 genes,~~. In fact, they have ~~where there is~~ only one intron. GTPuNGT sequence and PyAG sequence were conserved in 5′ and 3′ ends, respectively, of the gene encoding L41 protein of *Phaffia rhodozyma* ~~L41 gene~~; this conserved sequences ~~have~~ were also reported in the intron of actin of *Phaffia rhodozyma* ~~actin introns~~. The ~~*Phaffia rhodozyma*~~ L41 gene encodes ribosomal protein comprising 106 amino acids, and most notably, proline at position 56 is identified to the amino acid residue responsible for the sensitivity to cycloheximide. The genomic DNA sequence of the gene encoding L41 protein of *Phaffia rhodozyma* ~~L41 gene~~ was registered in GenBank on May 19, 1997, with accession NO. AF 004672 (see FIG 1 and SEQ ID NO: 15).

Example 2: a gene encoding mutant L41 protein having ~~C~~cycloheximide-resistant ~~L41 gene~~ activity

To confer the cycloheximide-resistance on the genen encoding the L41 protein~~gene~~, we performed a~~the~~ site-directed mutagenesis which resulted in the amino acid substitution~~converting~~ proline at position 56 with~~to~~ glutamine. Particularly~~Specifically~~, mutagenesis was carried out with the QuickChange in vitro mutagenesis kit (Stratagene) as described in the manufacturer's instructions with complementary mutagenic primers corresponding to amino acids 52 to 59 represented~~and described~~ by SEQ ID NO: 9 and 10. ~~Digested from the 8.0 kb fragment in Example 1, the~~ The 2.2-kb *Sal*I fragment digested from the 8.0-kb fragment

in Example 1 was replaced with the mutated fragment.

Example 3: The isolation of ribosomal DNA

Ribosomal DNA (rDNA) in ~~this~~ the present invention was exploited to enhance the integration efficiency of foreign DNA into *Phaffia rhodozyma* genomes. To clone the rDNA fragment, two pairs of PCR primers, ~~described~~ represented by SEQ ID NO: 11, 12 (corresponding to 18S rDNA part) and 13, 14 (corresponding to 28S rDNA part), were designed from the known partial rDNA sequence of *Phaffia rhodozyma*.

By PCR with these two pairs of primers, two DNA fragments were obtained.~~,~~ ~~one~~One of ~~which~~ those was 1.5-kb fragment containing the 5.8S rDNA NTS (~~;~~ non-transcription spacer) region with the primers represented~~described~~ by SEQ ID NO: 11 and 14, and the other ~~of which~~ was 6-kb fragment containing the 5S rDNA NTS region with the primers represented~~described~~ by SEQ ID NO: 12 and 13.

Two DNA fragments were used as a probe for cloning the rDNA unit in genomic Southern blot analysis, followed by the construction of minilibrary, as described in Example 1. Multiple rounds of Southern blot hybridization identified an 8.5-kb *Hin*dIII fragment, which was cloned and ~~whose~~ identity thereof was confirmed by partial sequencing. A 730-bp *Xho*I and *Xba*I fragment of the 8.5-kb fragment, which spans NTS region between 5S and 18S rDNA, was subcloned in pBluescript and the resulting vector was designated as pTPR4. ~~The~~ A sequencing of pTPR4 enlightened that the cloned rDNA fragment showed ~~much~~ high homology with

5.8S and 25S rDNA region of *Candida neoformans*, a member of Basidiomycetous yeasts including *Phaffia rhodozyma*. The 730-bp nucleotide sequence of *Phaffia rhodozyma* rDNA gene was registered in GenBank on July 28, 1997, with accession NO. AF 016256.

Example 4: The construction of recombinant vector for transforming *Phaffia rhodozyma*

To construct recombinant vectors for transforming *Phaffia rhodozyma* efficiently, we ~~exploited~~ constructed pTPL5 vector containing the gene encoding mutated L41 protein ~~gene~~ prepared in~~of~~ Example 2 and pTPR4 vector containing the rDNA fragment prepared in ~~of~~ Example 3 (see FIG 2). Particularly, pTPLR1 which is a recombinant vector ~~we constructed pTPLR1 vector~~ for transforming *Phaffia rhodozyma*~~,~~ was cunstructed using the 3.7-kb fragment of pTPL5 as a cycloheximide-resistant marker and the 730-bp rDNA fragment of pTPR4 as a targeting sequence whereby~~into~~ a forein DNA is integrated into *Phaffia rhodozyma* genome with multicopy. The 3.7-kb *Xba*I-*Sal*I fragment of pTPL5 containing the gene encoding a mutated L41 protein ~~gene~~ was treated with the Klenow enzyme and inserted into the *Bal*I site of pTPR4. The resulting plasmid, pTPLR1 (see FIG 3), was introduced into *E. coli* DH5α strain, and the transformed *E. coli* strain was deposited in Korean Collection for Type Cultures (KCTC) on October 21, 1998 (accession NO: KCTC 0535BP).

We also constructed a plasmid, pTPLR2, which has the reverse direction agaist~~of~~ the ~~expressed~~ coding sequences. The pTPLR1 and pTPLR2 vectors were digested

with *Sma*I or *Bgl*I-*Kpn*I restriction enzymes, before the vector was brought to the transformation and integrated into the rDNA region of *Phaffia rhodozyma* genome.

<u>Example 5: The transformation of *Phaffia rhodozyma* with pTPLR1 vector</u>

To transform *Phaffia rhodozyma* with the pTPLR1 vector efficiently, we developed the transformation method, which is based upon the method for transforming a Basidiomycetous yeast, *Cryptococcus neoformans* (Varma *et al., Infect. Immun.*, 60, 1101, 1992) but optimized for *Phaffia rhodozyma*. Electroporation procedure was employed in the process of ~~this~~ <u>the present</u> invention. Particularly, *Phaffia rhodozyma* cells from a log-phase cluture in 50 ml of YM medium were harvested by centrifuge at 3,000 rpm for 10 minutes, then washed twice with equal volume of electroporation buffer (270 mM sucrose, 10 mM Tris, 1 mM $MgCl_2$, pH 8.0) containing 1 mM dithiothreitol (~~,~~ DTT), and resuspended in the electroporation buffer without DTT. The linearized plasmid pTPLR1 (200 ng) was mixed with a 50 µl aliquot (approximately 2×10^7 cells) of the cell suspension, and transferred to a cuvette (0.2-cm electrode gap; Bio-Rad, USA). <u>Sets of</u> ~~We performed~~ electroporation <u>were performed</u> (Gene Pulser II; Bio-Rad, USA) under the various ranges of electric pulse (0.8 to 1.2 kV), internal resistance (400 to 800 Ω) and capacitance (25 to 50 µF). The electroporated cells were resuspended in 1 ml of YM medium and transferred to a test tube for incubation. After being shaken for 12 to 16 hours at

23°C, cells were spread on YM agar medium containing 10 μg/ml of cycloheximide and incubated at 23°C for 4 to 5 days.

Figure 4 shows the relationship between the condition of electroporation and the transformation efficiency or cell viability. The transformation efficiency was mainly dependent on the capacitance, preferably of 50 μF rather than 25 μF. In summary, more transformants were produced when an electric pulse of 0.8 kV was delivered and internal resistance of 600 Ω was set with a capacitance of 50 μF, generating pulse lengths of 18 to 20 ms. Under such a condition, approximately 30% of cells survived, and transformation efficiencies of 800 to 1000 transformants per μg of DNA could be routinely obtained with pTPLR1 linearized either by *Sma*I or by *Bgl*I-*Kpn*I.

Using the optimized process, we transformed *Phaffia rhodozyma* with various vectors and observed the colony formation on the YM agar medium containing cycloheximide.

Interestingly, there was no transformant with pTPLR2 in any condition, suggesting that L41 gene is expressed only when the transcriptional direction of the integrated L41 gene is the same as that of rDNA.

Without the ~~restriction~~ linearization of pTPLR1 before transformation, no colony was formed. This may result from the fact that rDNA does not have the autonomous replication sequence (~~;~~ ARS) or its similar function.

A vector carrying a gene encoding a mutated L41

protein having cycloheximide-resistant activity~~L41 gene~~ but not containing rDNA sequence, was introduced into *Phaffia rhodozyma*. In this case, a few colonies were observed. We suspected that the mutated L41 gene in the vector would replace endogenous L41 gene in the genome, rather than be integrated in directed position.

In addition, we transformed *Phaffia rhodozyma* with a vector ~~in which~~wherein the promoter of L41 gene was deleted, and observed transformed colonies. The Southern blot analysis of this transformant showed the same hybridization pattern as that of nontransformant control. This indicates that ~~in this case also~~ the a substitution has ~~transplacement has~~ occurred in this case, rather than ~~be~~ an integrationed in the directed position.

Example 7: Southern blot analysis of the transformants

To assess the stability of the introduced foreign DNA in *Phaffia rhodozyma* genome according to ~~this~~ the present invention, ~~we performed~~ a Southern blot analysis of genomic DNA, which is prepared from pTPLR1 transformants or nontransformant control was performed (see FIG 5). Particularly ~~T~~the genomic DNA was digested with *Sma*I or *Eco*RI enzyme, and the 2.2-kb *Sal*I fragment of pTPL2 was used as a probe in the hybridization. The intensity of colored band was measured by the scanning densitometer (Model GS-700 Imaging Densitometer, Bio-Rad, USA).

Southern blot analysis, ~~in which~~wherein genomic DNA of transformants was digested with *Sma*I, showed two colored bands at 9.0-kb and 4.1-kb. A signal at 9.0-kb

is observed both in a nontransformant control and in the transformants, indicating that this band originated form the endogenous gene encoding L41 protein of *Phaffia rhodozyma*~~ L41 gene~~. A much stronger signal at 4.1-kb also was detected in transformants, but not in the control. This was identical with the result of~~expected from~~ the restriction map of the transforming plasmid (see FIG 6). The size and relative intensity of signal at 4.1-kb suggested that multiple copies (approximately, 7 copies) of the transforming plasmid had been integrated.

In another Southern blot analysis with *Eco*RI digestion, two bands at 5.8-kb and 2.8-kb were found only in transformants (see FIG 5). The 5.8-kb band originated from a 3.2-kb rDNA fragment and a 2.6-kb L41 gene fragment, and the 2.8-kb band originated from a 1.7-kb rDNA fragment and a 1.1-kb L41 gene fragment. Integration ~~probably~~ may occur~~s~~ as diagrammed in Figure 6.

These results were reproducible in Southern blot analysis with rDNA probe. Most importantly, copy number did not decrease after a prolonged cultivation in YM medium with or without cycloheximide, indicating that the transforming plasmid was integrated into the chromosome and maintained stably.

INDUSTRIAL APPLICABILITY

As shown above, the vector~~s~~ for transforming *Phaffia rhodozyma* of the present invention comprises rDNA and a gene encoding a mutated L41 protein having cycloheximide-resistant activity~~L41 gene~~, which is~~are~~

useful for a~~the~~ stable integration of foreign DNA into host genome and for a~~the~~ convenient selection of transformants, respectively. ~~These~~The vector~~s~~ of the present invention is~~are~~, therefore, applicable to the transformation of yeast cells including *Phaffia rhodozyma*, in combination with the process for transforming yeast cells ~~process~~ of ~~this~~the present invention, wherein the yeast cells are transformed through the optimized electroporation.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

ABSTRACT OF THE DISCLOSURE

The present invention is directed~~relates~~ to a ~~transforming~~ recombinant vector for transforming yeast and a process ~~of~~ for transforming~~ation~~ yeast thereby, more ~~specifically~~ particularly to a ~~trasnsforming~~ recombinant vector comprising a gene encoding a mutated L41 protein having cycloheximide-resistant activity~~gene~~ and a ribosomal DNA. The recombinant~~transforming~~ vector and the process for transforming ~~process~~ thereby of the present invention is applicable to the efficient and stable integration of desired foreign DNA into yeast genome, thus providing useful tools for the production of a natural pigment, astaxanthin.